A Thesis for the Degree of Master of Science

A protective effect of inulin on porcine intestinal epithelial cells with damage induced by deoxynivalenol

Deoxynivalenol에 의한 돼지 장상피세포 손상으로부터 이눌린의 장벽보호 기능

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By

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농 학 석 사 학 위 논 문

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이 논문을 농학 석사학위논문으로 제출함

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Summary

Inulin, when passing through the stomach and duodenum undigested, is known as highly nourishing to the colonic bacterial flora that convert inulin into short-chain fatty acids. Although the effects of inulin mostly as prebiotics in pigs have long been investigated, its action mechanism on porcine intestinal epithelial cells is not well understood.

In the present study, protective role of inulin was examined on porcine intestinal epithelial cells (IPEC-J2) with/without damage induced by deoxynivalenol (DON). The results showed that inulin enhanced barrier function by inducing the increase of ZO-1, occludin, and claudin-3 in both mRNA and protein levels. Furthermore, inulin activated Akt which induce the expression of tight junction proteins. Pre-incubation of IPEC-J2 with inulin attenuated DON-induced decline of para-cellular and trans-cellular permeability and disruption of tight junction proteins. Moreover, inulin pretreatment inhibited DON-induced IL-8 expression suggesting that inulin may overwhelm the DON-induced inflammation.

Collectively, the present study suggests that inulin directly strengthens the intestinal barrier function by increasing the tight junction proteins via Akt-dependent manner and decreasing the pro-inflammatory condition in damaged porcine epithelial cells.

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List of Abbreviations

BCL-2 B Cell Lymphoma-2

CLN Claudin

CON Control

DON Deoxynivalenol

DP Degree of polymerization

ELISA Enzyme linked immunosorbent assay

FAK Focal adhesion kinase

FBS Fetal bovine serum

FOS Fructo-oligosaccharide

GOS Galacto-oligosaccharide

Hck Hematopoietic cell kinase

IEC Intestinal epithelial cell

IL Interleukin

Inu Inulin

IPEC-J2 Intestinal porcine epithelial cell line-J2

MAPK Mitogen-activated protein kinase

mRNA Messenger RNA

MTT 3-(4,5-dimethylathiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells

OCLN Occludin

PBS Phosphate buffered saline

PKC Protein kinase C

PKR Protein kinase RNA-activated

RT-PCR Real-time quantitative polymerase chain reaction

SCFA Short chain fatty acid

TEER Transepithelial electrical resistance

TJ Tight junction

TLR Toll-like receptor

ZO Zonula occluden

I. Review of Literature

1. Inulin

1.1 Potential role of prebiotics

The prebiotics have been defined as a non-digestible oligosaccharides which selectively induces the growth/activity of probiotics and beneficially act on the host's health [1]. The increased probiotics can convert prebiotics into short-chain fatty acids (SCFAs), which nourish colon cells and paneth cells, and offer various health benefits. Thus, prebiotics is used for progress digestive health, prevent the mucosal inflammation, and help control diabetes [2]. The major sources of prebiotics are derived from various food such as asparagus, banana, chicory root, onion, and milk [3]. Nowadays, there are fructooligosaccharide (FOS), galactooligosaccharide (GOS), xyloseoligosaccharide, and inulin have been commonly designated to prebiotics [4]. Specifically, inulin structurally contains β-D fructan that is bound by β-2,1 glycosidic bonds with glucose unit at the end [5]. By the number of glycoside bonds unit, which is called as degree of polymerization (DP), That is divided into FOS (2<DP <9, average DP = 4.8) and inulin (2<DP<60, average DP = 12) [6].

The characteristic of inulin is well recognized as a prebiotic with

beneficial effects on human or animal by selectively upregulating the growth probiotics for instance, *Bifidobacterium* in the large intestine.

1.2 Beneficial effects of inulin in pigs

Feed components have a crucial role for the productivity and growth performance in pigs. Thus, researchers concentrated on feeding and gut health. Inulin, which can be used as a food supplementation, has been suggested that have beneficial role in the health. Previous studies identified that feeding with inulin for growing porcine increased daily body weight gain [7, 8]. Moreover, last period diet containing inulin reduced the skatole levels of boars, which is sex-specific odor that is an important factor for consumer, in intestinal contents and adipose tissue [9, 10].

A recent study demonstrated inulin has the anti-inflammatory response in the gut-associated lymphoid tissue. The pigs fed with inulin significantly enhanced interleukin (IL)-10 on mitogen-activated intraepithelial lymphocytes isolated from distal jejunal section than control [11]. Furthermore, inulin has shown protective effects against external infection of microbiomes. It has been suggested that inulin could inhibit infection with pathogens such as enterotoxigenic *Escherichia coli*. Supplementation with inulin recovered the post-weaning diarrhea by improving the fecal

consistency and reducing the incidence for post weaning diarrhea [12]. It has also proven that inulin inhibited the parasites against *Oesophagostomumdentatum* and *Trichuris suis* post infection [13, 14].

1.3 Inulin-induced cellular activity

It has been demonstrated that inulin has an ability to regulate cytokines expression of immune cells and strengthen the barrier function of intestinal epithelial cells. Aside from immune regulation via microbial interaction to produce SCFAs, inulin may directly regulate immune system. Previous study showed that FOS increased the toll-like receptor (TLR)-4 mediated IL-10 production on monocyte derived dendritic cells [15]. Moreover, in the intestinal epithelial cells, IL-8 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) were decreased by FOS treatment in Caco-2 cells [16, 17]. This study suggested that peptidoglycan recognition protein 3 reducing the expression of pro-inflammatory cytokines that was increased by inducing the nuclear receptor, peroxisome proliferator-activated receptor-gamma

Furthermore, inulin protects barrier integrity via TLR2-downstream signaling, which could strengthen the barrier, in damaged T84 human intestinal epithelial cells [18]. Additionally, inulin pretreatment with the

infection of *E. coli* (EHEC) O157:H7 to human Caco2-Bbe1 epithelial cells attenuated EHEC-mediated disruption of barrier. A current paper demonstrated that inulin has the protective effect, increasing of expression of tight junction protein, through protein kinase C (PKC)-δ-dependent signaling [19].

2. Deoxynivalenol

2.1 Definition and characteristics

Fusarium mycotoxins are one of the largest groups of mycotoxins that are synthesized by many species of fungi, mainly by Fusarium (*F. graminearum* and *F. culmorum*) in the contaminated grains including corn, wheat, oats, and rye [20]. Mycotoxins can affect the toxic effects in domestic animals in field, that lead to reduction of body weight gain, low levels of fertility which can lead to cause the death [21].

Among the various myctoxins, Deoxynivalenol (DON) is known as vomitoxin (its chemical name:12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-on) due to its strong emetic effects [22]. It contains 3 free hydroxyl parts (-OH), which are known to be associated with its toxicity (Fig. 1).

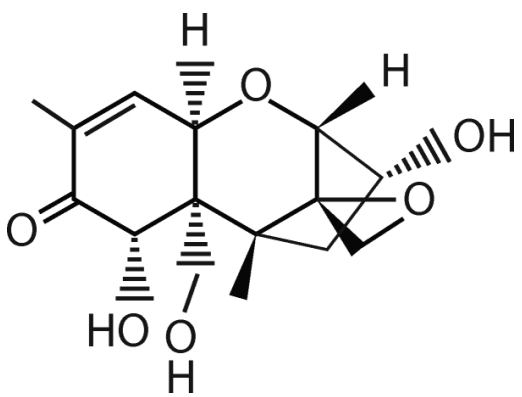

Figure 1. Chemical structure of deoxynivalenol.

The negative effects from DON induce short-term nausea, headache, vomiting, dizziness, abdominal pain, diarrhea in public health which is not the threating level [23]. On the other hand, there is less productivity by DON in the domestic animal. It resulted in the genotoxic effects of broiler chickens and reducing of performance in dairy cattle [24, 25].

One of the physicochemical characteristics of DON is to resist high temperature, that allow existing in processed food. Numerous studies have documented that DON is heat-stable in the range of 170°C to 350°C [20]. Therefore, processed feed may include DON and contaminated crops consisting DON can induce considerable economic losses for agricultural and livestock industry.

2.2 Mechanism of disruption on intestinal barrier

DON not only binds eukaryotic ribosomes and interferes with translation in the cytoplasm of the cell but also activates intracellular protein kinases that mediate selective gene expression and apoptosis, ultimately contributing to downstream pathologic results [26]. In terms of *in vivo* trial, DON leads to immune stimulation or inflammation, anorexia, reduction of weight gain, and tissue loss [27].

As shown in Fig. 2, double-stranded RNA-activated protein kinase (PKR) and hematopoietic cell kinase (Hck) induce the activation of the DON-induced mitogen-activated protein kinases (MAPK) components resulting in ribotoxic stress response. [28, 29]. Activated MAPKs could induce transcription factor activation which affects to increase of pro-inflammatory genes that lead to apoptosis and chronic and immune-toxic effects. [30]. Furthermore, MAPK impairs the barrier function by decreasing the tight junction proteins [31]. For instance, DON induced changes of porcine jejunal histology by shortening the villi and lysis of enterocytes [32]. DON showed an increase of paracellular permeability by reducing tight junction proteins such as claudin (CLN) expression including CLN3 and CLN4 in human and porcine intestinal epithelial cells (IECs) [31, 33, 34].

It has been suggested that highly dividing cells such as IECs or immune

cells are particularly sensitive to DON. The action mechanism of DON is summarized and depicted in Fig. 2.

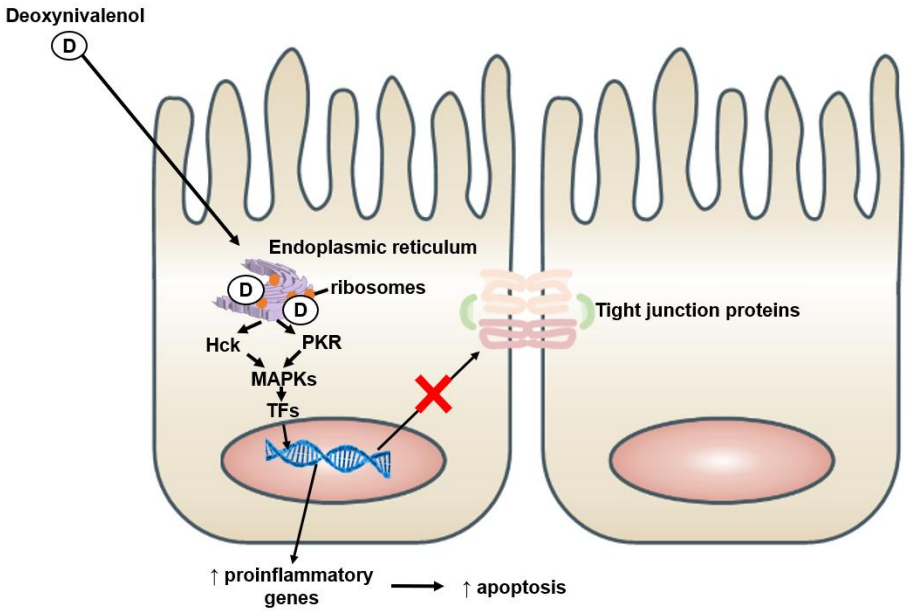

Figure 2. Molecular mechanism of deoxynivalenol.

2.3 Toxic effects in pigs

Among the frequently consumed meat in human, the demand of pork has been increased worldwide by consumers. Thus, it is important to make high productivity of pork [35]. However, monogastric animals as like pigs are more susceptible to DON, as they lack a microbiota to degrade mycotoxin before their absorption [36]. Hence, reduced feed consumption and poor performance have been reported in swine, even when fed diet containing a low amount of DON (1-5 ppm). At the higher toxin levels (>5 ppm) in feed, changed serum composition, denial of feed, and immunotoxicity have been reported in pigs than other species [37]. The symptoms of pigs fed with DON include abdominal distress, discomfort, diarrhea, vomiting, and gastrointestinal bleeding [38, 39]. Thus, detoxifying the DON is on the high demand to make good quality of meat in pig industry.

3. Tight junction proteins

3.1 Characterization of intestinal tight junction proteins

The intestinal mucosal surface in the gut is composed of predictable 10 to 100 trillion organisms [40]. As linkage with dense and numerous communities of microbiota, it is regarded as the first line of defense from penetration or invasion of pathogens [41]. In the intestinal barrier, it prevents the entrance of foreign antigens, toxins from external stimuli and has a selective permeability to nutrient absorption including amino acids, peptides, fatty acids, minerals and vitamins [42]. Tight junction proteins play a role as cell-cell association of epithelial barrier to inhibit the unwanted penetration of various molecules and materials and to maintain the selective permeability. These proteins contain the three major components: Zonula occludens, occludins and claudins family [43]. If the tight junction is disrupted, it leads to increase of permeability and activation of mucosal immune responses [44]. Therefore, the expression and maintenance of tight junction proteins are important for forming an intestinal barrier function.

3.2 Zonula occluden family

Zonula occluden (ZO) was found as TJ-specific protein including, ZO-1, ZO-2, and ZO-3. Their multi-domain has a scaffold structure for intracellular bridging to the protein. The N-terminal region of ZO protein is bound to occludin or claudin, and the C-terminal region interacts with actin filaments [45]. ZO-1 is found to be localized to the nascent cell-cell contacts. Function of ZO-1 was reported by using ZO-1 deficient cells that showed delayed assembly of other TJ proteins such as claudins and occludin. Therefore, ZO has been indicating the importance in regulation of tight junction protein assembly [46].

3.3 Occludin family

Occludin (~ 65 kDa) was firstly designated as a TJ protein for integral membrane [47]. It is a tetra-trans membrane protein with a long C-terminal and four transmembrane domains, two extracellular loops, and one intracellular loop. Occludin was revealed that decrease the macromolecules flux. However, small ions are not blocked by the occludin [48]. The function of occludin is still unclear, but many studies have suggested that it could play a role on forming structure for the permeability of tight junction proteins [49].

3.4 Claudin family

Claudin family has been reported to contain 27 members. Claudins (22 kDa) are membrane proteins and consist of one intracellular and two extracellular loops, and C-terminal and N-terminal cytoplasmic domains [50]. It has been shown that claudins are an important factor to tighten the junctions of epithelial barrier [51]. Although claudin family has a similar structure, they can be classified according to their function based on controlling the permeability (Table 1). One group including claudin 1, 3, 4, 5, 8, 9, 11, and 14 forms the barrier, and at the same time down-regulates the permeability, while the other group, claudin 2, 7, 12, and 15, forms the channel pore, and up-regulates permeability [52]. The functions of claudins were identified by observing claudin-1 knockout mice that died within 24 hours after birth because of that mice have impaired skin barrier resulting in significant loss of fluid and ions [53]. Therefore, claudins play an important role that forms the barrier and channel pores. In addition, it also absorbs the nutrients, fluid and ion transportation.

Table 1. The major types of claudins.

Claudin sub-types	**Changes in paracellular permeability**
Claudin 2, 7, 12, and 15	Increase

Claudin 1, 3, 4, 5, 8, 9, 11, and 14	Decrease

4. Akt signaling and barrier protection

4.1 Akt signaling on the intestinal epithelial cells

The Akt, also known as protein kinase B, is serine/threonine specific protein kinase that has a role in cell proliferation, apoptosis, and cell migration [54]. Akt signaling could be activated by receptor tyrosine kinase, G-protein receptor or toll-like receptor (TLR). That receptors have associated with various down-stream signaling including B cell lymphoma (BCL)-2 and p70S6K. In detail, in the cellular mechanisms of Akt, focal adhesion kinase (FAK) is the upstream of Akt that regulates the survival and increase the expression of tight junction proteins [55, 56]. Activated Akt phosphorylated the p70S6K, which directly modulates intestinal protein synthesis [57]. Furthermore, another downstream molecules of Akt, BCL-2 has a role of anti-apoptosis [58]. That passes on appropriate signals to cells of immune system. Moreover, recent studies have provided important insight host defense mechanisms to maintain functional barrier integrity of the intestinal epithelium through Akt.

4.2 Barrier protective effect of Akt

It is demonstrated gastric mucosal epithelium revealed that Akt signaling may enhance barrier integrity. Moreover, that enhances the cell survival and proliferation [59, 60] as well as prevents cell death [61]. Previous studies showed activated Akt was observed that may enhance the tight junction proteins and inhibit cell death from damaged IECs [34, 60]. As shown in Fig. 3, the Akt signal is suggested that it is important for enhancement of tight junction proteins in intestinal epithelial cells [59, 60, 62].

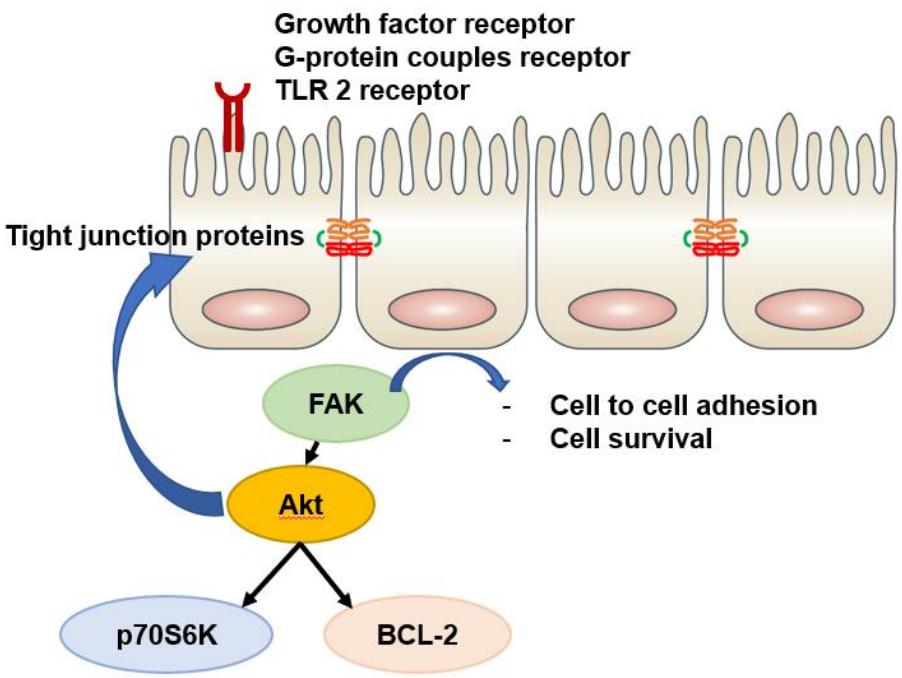

Figure 3. Epithelial cells increase barrier function via activation of the Akt.

II. Introduction

Gastrointestinal health is a central for the productivity of domestic animals, and thus researchers have concentrated on gut homeostasis and intestinal integrity to maintain and preserve the barrier function [63]. Especially, balanced microbiota population is an inevitable element for a healthy condition of intestine. To maximize animal productivity, there are probiotics, living microorganisms, when administered in appropriate amounts that will cause a healthy gastrointestinal environment in the host [64]. On the other hand, prebiotics could help to thrive the certain gut microbiota and to enhance the growth performance of domestic animals [65, 66]. They appeared to have a beneficial effect on gut health through positively regulating the composition of probiotics in the large intestine [67].

Inulin, one of the prebiotics, is defined as soluble, non-digestible and naturally occurring oligosaccharides that beneficially affect host health through increasing the composition of probiotics such as *Bifidobacterium* and *lactobacillus*. As a result, increase in the production of short-chain fatty acids as byproduct [32] that is used by colonocytes as an energy source to produce the mucus [47]. In addition, inulin has been proposed to influence

direct function of gut epithelium. Inulin appears to strengthen the barrier function. The studies have shown that inulin induced the decrease of intestinal permeability, enhanced assembly of tight junction together with reduction of pro-inflammatory cytokines in a PKCδ- or TLR2-dependent manner [18, 68]. Moreover, piglets fed with a diet containing inulin positively affected the morphology of small intestine, resulting in better development and thickening of the intestinal villi when compared with control [69]. However, direct effect of inulin on porcine IECs and its molecular and cellular mechanism of action remains unclear.

Deoxynivalenol (DON), mycotoxins derived from *Fusarium* spp [70], has a high tolerance level against heating and during feed processing [6]. Detrimental effects of DON in pigs include gastrointestinal irritation, necrosis of the IECs, diarrhea, vomiting in conjunction with feed refusal and decreased body weight gain [71]. More specifically, as DON activates intracellular protein kinases for selective gene expression and apoptosis leads to break down the barrier integrity of the intestinal epithelium and increases the level of pro-inflammatory cytokines [29, 72, 73].

The aim of the present study is to define the cellular and molecular mechanism of inulin on barrier function and integrity of IECs. Furthermore, the protective effect of inulin on porcine IECs was evaluated in the cells

with a damage induced by DON.

III. Materials and Methods

IPEC-J2 culture

Non-transformed intestinal (jejunum) porcine epithelial cell line, IPEC-J2 (DSMZ, Germany) was cultured in the Dulbecco's modified Eagle medium containing Nutrient Mixture F-12 (Ham) with GlutaMAX™ supplement (DMEM/F-12) (Gibco Life Technologies, Grand Island, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% insulin-transferrin-selenium-X, antibiotics (all from Invitrogen, Carlsbad, USA) and 2% of 16 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES, Gibco Life Technologies) in an incubator with atmosphere of 5% CO_2 at 39°C. The medium was replaced every 3 days.

Inulin and DON Treatment

To evaluate the effect of inulin on the barrier function, differentiated IPEC-J2 were washed with phosphate buffered saline (PBS) and treated with 0, 10, 100 or 1000 μg/mL of inulin for 24 h. To select the appropriate dose, IPEC-J2 cell monolayer was treated with 0, 2.5, 5, or 10 μM of Deoxynivalenol (DON; Sigma-Aldrich) for 24, 48, or 72 h. To determine the

protective function of inulin, IPEC-J2 were pre-treated with 1000 μg/mL of inulin for 24 h, and then with 5 μM of DON for 48 h.

Transepithelial electrical resistance assay

IPEC-J2 were seeded on 1.12 cm^2 polyethylene terephthalate membrane inserts with pore size, 0.4 μm (Corning, NY, USA) while the basolateral side was filled with 1 mL of DMEM media. During the cell growth and differentiation, the medium in both compartments was replaced every three days. After the differentiation, DON (0, 2.5, 5, or 10 μM) was treated for 24 to 72 h depending on the purpose. To identify the protective effect of inulin, the cells were pre-treated with inulin for 24 h and then, treated with or without DON for 48 h. Transepithelial electrical resistance was measured by an epithelial tissue voltohm meter (EVOM2; World Precision Instruments, USA) and its value was expressed as the percentage of initial value or percentage of control, that is baseline relative to the untreated wells.

Real-time qRT-PCR

Total RNA was isolated using the Tri-RNA reagent (Favorgen, Perth, Australia) according to the manufacturer's instruction and reverse-transcribed for generation of complementary DNA (cDNA) using oligo-dT

primers (Bioneer, Daejeon, Korea). Real-time qRT-PCR was performed using a StepOne Plus real-time PCR system (Applied Biosystems, Foster city, CA, USA). The PCR reaction was carried out in a 96-well reaction plate with 10 μL SYBR® green PCR master mix, 0.5 μL specific primers, 1 μL cDNA template, and 8 μL nuclease-free H_2O. Thermal cycles consisting of 2 min at 50℃, 10 min at 95°C, 15 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C were repeated 40 times. The nucleotide sequences of specific primers for Zonula Occluden (ZO)-1 (forward primer: 5′-GCAAAAATCATAAGGAGGTCGAA -3′, reverse primer: 5′-TGGCTAACTGCTCAGCTCTGTT-3′), Occludin (OCLN) (forward primer 5'-CCTCAGGCAGCCTCATTACAG-3', reverse primer: 5′-GGGAGCCCGTTTTGAAGAC-3′), Claudin-1 (CLN1) (forward primer: 5'-CTGGGAGGTGCCCTACTTTG-3', reverse primer: 5'-GGGCCTTGGTGTTGGGTAA-3'), Claudin-3 (CLN3) (forward primer: 5'-TGTCCGTCTATCCGTCCGTC-3', reverse primer: 5'-ATCCGCGCTGTGATAATGCT-3') and interleukin (IL)-8 (forward primer: 5'-GCTCTCTGTGAGGCTGCAGT-3', reverse primer: 5'-AAGGTGTGGAATGCGTATTT-3') were used. Relative quantification of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Target gene expression was normalized to the mRNA level of porcine beta-actin (forward primer:

5′-GATGAGATTGGCATGGCTTT-3′, reverse primer: 5′-CACCTTCACCGTTCCAGTTT-3′).

Western blot

Confluent IPEC-J2 treated with inulin (0, 10, 100, or 1000 μg/mL) were treated for 24 h. The damage was induced by using 5 μM of DON on the confluent IPEC-J2 pretreated with or without 1000 μg/mL of inulin. In other experiment, 1 μM of Akt inhibitor (Sigma-Aldrich) was treated for 24 h prior to inulin treatment. Then, the cells were washed with cold PBS and lysed in a lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing protease inhibitor, followed by a quantitation of protein using Micro BCA kit (Thermo Fisher Scientific, Rockford, USA). The same amount of protein extracts was loaded in 10% Tris-glycine polyacrylamide gels and electrophoresed. Then, the proteins were transferred onto a polyvinylidene difluoride (PVDF) microporous membrane for 2 h at 4°C and blocked with 5% skim milk in TBS-T (20 mM Tris HCl, 100 mM NaCl, 0.05% Tween 20) for 60 min. The blot was incubated with rabbit anti-claudin-3 (CLN3), -occludin (OCLN), -zonula occluden-1 (ZO-1) antibodies (Invitrogen, Carlsbad, CA, USA), anti-p-Akt (ser), -p-p70S6K, -p-Focal Adhesion Kinase (FAK), and -B Cell

Lymphoma-2 (BCL-2) or mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) overnight. Subsequently, the membrane was washed and incubated with goat anti-rabbit or anti-mouse IgG-HRP (Santa Cruz Biotechnology) for 1 h. The target protein was visualized with enhanced chemiluminescence system (GE Healthcare, Waukesha, WI, USA), followed by analysis using ChemiDoc XRS (Bio-Rad, Hercules, CA, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

IPEC-J2 at 1×10^5 cells/mL of IPEC-J2 in DMEM/F12 media were seeded onto 96-well plates. At the confluent stage, the cells were pretreated with or without inulin for 24 h and treated with DON for 48 h. 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/mL in PBS; Sigma) was added to each well for 2 h, and then media discarded. One hundred microliter of DMSO (Sigma-Aldrich) was added to each well and shaken for 5 min to solubilize the formazan formed in the viable cells. Absorbance was measured at 595 nm using a microplate reader (Molecular devices, Sunnyvale, CA, USA)

Enzyme-linked immunosorbent assay

The cell culture supernatants were collected from the confluent IPEC-J2 at 24 h after the treatment. The concentration of IL-8 was determined by using enzyme-linked immunosorbent assay Duoset kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instruction. In brief, the cytokine capture antibody was coated on 96-well immuno-plate purchased from Nalgene Nunc International (Rochester, NY, USA) and incubated overnight at room temperature. The plates were then washed with washing buffer (0.05% Tween 20 in PBS) for three times and blocked with blocking buffer (1% bovine serum albumin in PBS) for 1 h. After washing, non-diluted culture supernatants and respective standard IL-8 were added into each well and incubated for 2 h followed by 2 h incubation with detection antibody conjugated with biotin. Specific binding was examined using streptavidin–HRP (R&D Systems) followed by the addition of the TMB substrate (Sigma-Aldrich). The reaction was stopped with 50 μL of 2 N H_2SO_4. The amount of cytokines was measured at absorbance of 450 nm by using a microplate reader (Molecular devices).

Statistical analysis

Statistical analysis (one-way ANOVA with Tukey posttest or two-way ANOVA with Bonferroni posttest) was performed using the GraphPad Prism (version 5.01, GraphPad Software, San Diego, USA). Differences were considered significant if $P < 0.05$.

IV. Results

1) Inulin enhances barrier function in IPEC-J2 cells.

To determine potential cytotoxicity or cell death, IPEC-J2 were treated with inulin for 24 h. The result with the range of 10 to 1000 μg/mL showed that inulin-induced cell death or cytotoxicity was not observed (data not shown). Next, in order to examine potential change of barrier function, IPEC-J2 grown in trans-well were treated with various doses of inulin and the resistance was measured by using transepithelial electrical resistance (TEER). The results showed that inulin treatment increased the TEER in a dose-dependent manner (Fig. 4A). TEER was also enhanced in a time-dependent manner, which was significant ($P < 0.01$) at 24 h-post treatment (Fig. 4B).

To investigate whether increased epithelial barrier integrity was associated with changes of tight junction proteins, the mRNA levels of *ZO-1*, *OCLN*, and *CLN3* were measured. The results showed that 1000 μg/mL of inulin induced significant ($P < 0.05$) increase of the transcriptional level of *ZO-1*, *OCLN* and *CLN1* and *CLN3* (Fig. 4C).

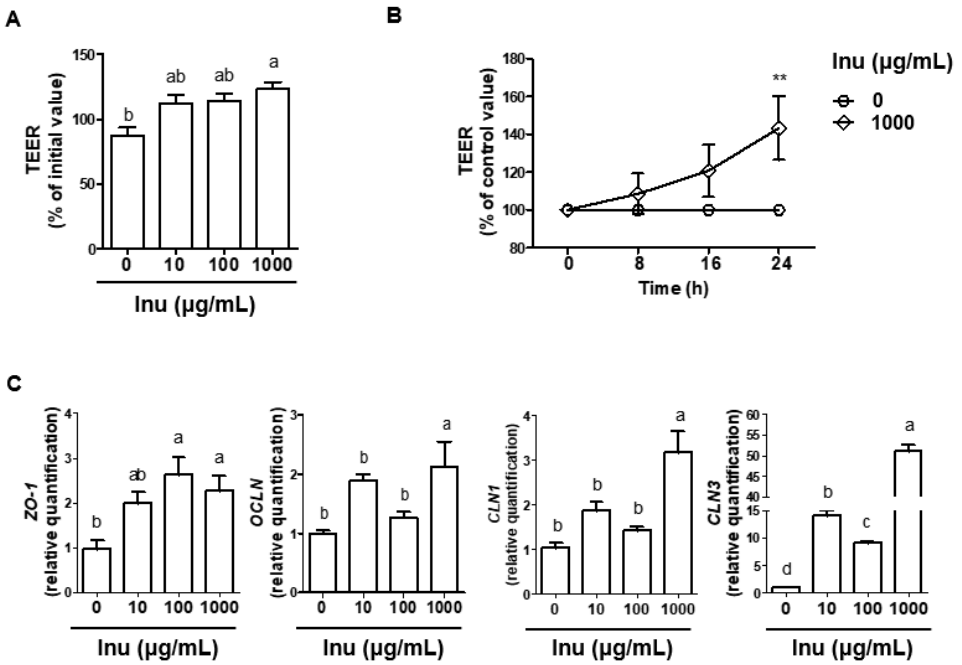

Figure 4. Inulin enhances barrier function in IPEC-J2 cells. Differentiated IPEC-J2 were treated with inulin (Inu) (A) at 0, 10, 100, or 1000 μg/mL for 24 h, or (B) at 1000 μg/mL for 0, 8, 16 or 24 h. TEER value was measured using voltohm meter. (A) It is depicted as a percentage of initial value. Different letters indicate significant differences at $P < 0.05$ between the groups. (B) The data are represented as a percentage of control value (n =3). ** indicates a significant difference at $P < 0.01$. (C) Confluent IPEC-J2 were treated for 6 h (n = 3). Real-time PCR was performed to determine the mRNA expression of ZO-1, OCLN, CLN1, and CLN3. All values are expressed as mean ± SEM. Different letters indicate significant differences at $P < 0.05$

between the groups.

2) Inulin increases tight junction proteins in IPEC-J2 cells.

To further examine the effect of inulin, the changes of tight junction proteins were measured by using Western blot assay. The results showed that IPEC-J2 treated with inulin increased tight junction proteins in a dose-dependent manner (Fig. 5). ZO-1 (Fig. 5A) and OCLN (Fig. 5B) were significantly ($P < 0.05$) increased. Similar to the mRNA level, the protein level of CLN3 was also significantly ($P < 0.01$) increased (Fig. 5C).

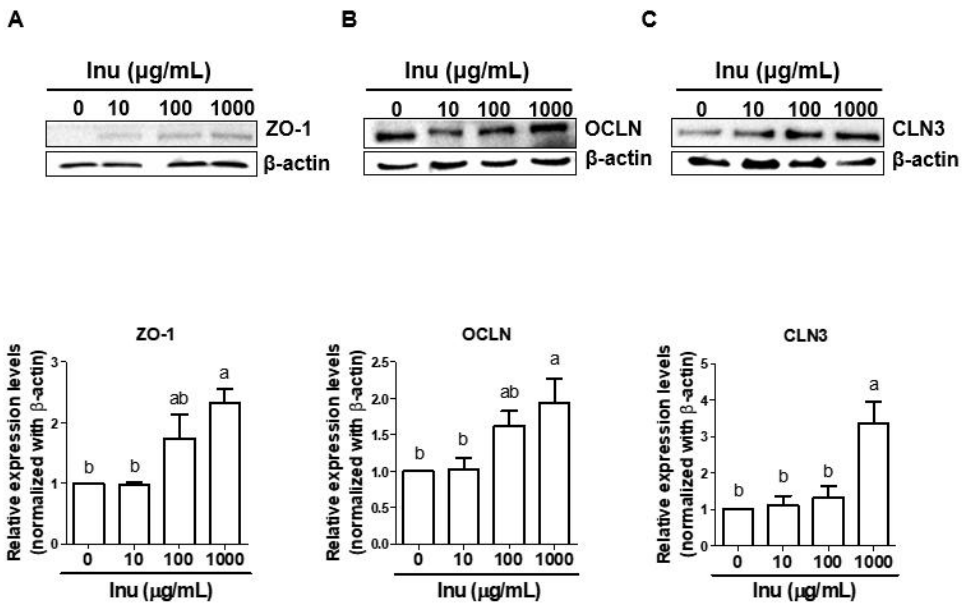

Figure 5. Inulin increases tight junction proteins in IPEC-J2 cells. Confluent IPEC-J2 were treated with inulin (Inu) at 0, 10, 100, or 1000 μg/mL for 24 h. The cells harvested were lysed, and the protein extracts were analyzed for (A) ZO-1, (B) OCLN, and (C) CLN3 by using Western blot assay. β-actin was used as an internal control. All values are expressed as mean ± SEM (n=3). Different letters indicate significant differences at $P < 0.05$ between the groups.

3) Inulin activates Akt signaling to increase the expression of tight junction proteins in IPEC-J2 cells.

It has been suggested that Akt is associated with the increase of tight junction assembly [74] in conjunction with p-FAK, p-p70S6K, and BCL-2 [34]. To evaluate whether Akt is involved in inulin modulation of tight junction protein, Akt signaling molecules were measured by Western blot assay. The results showed that all target proteins, such as p-FAK, p-p70S6K, p-Akt, and BCL-2 (Fig. 6A-E), were significantly ($P < 0.05$) increased in the IPEC-J2 with inulin. Furthermore, I investigated whether inulin-induced Akt signals were prevented by Akt inhibitor. The adverse effect of Akt inhibitor was checked by comparing between control and Akt inhibitor. There was no difference between control and Akt inhibitor. However, inulin treatment significantly down-regulated the expression of Akt signaling molecules in the presence of Akt inhibitor (Fig. 6A-E). Thus, these results suggested that inulin induced activation of Akt-associated molecules.

Next, to validate the effect of Akt in association with the barrier function, Akt signal was inhibited in IPEC-J2 treated with inulin and examined the alteration of tight junction proteins. Inulin-mediated tight junction proteins, ZO-1 (Fig. 6F), OCLN (Fig. 6G) and CLN3 (Fig. 6H)

were significantly down-regulated.

Collectively, these results suggested that inulin strengthens the barrier function that was in relation to the downstream of Akt signaling.

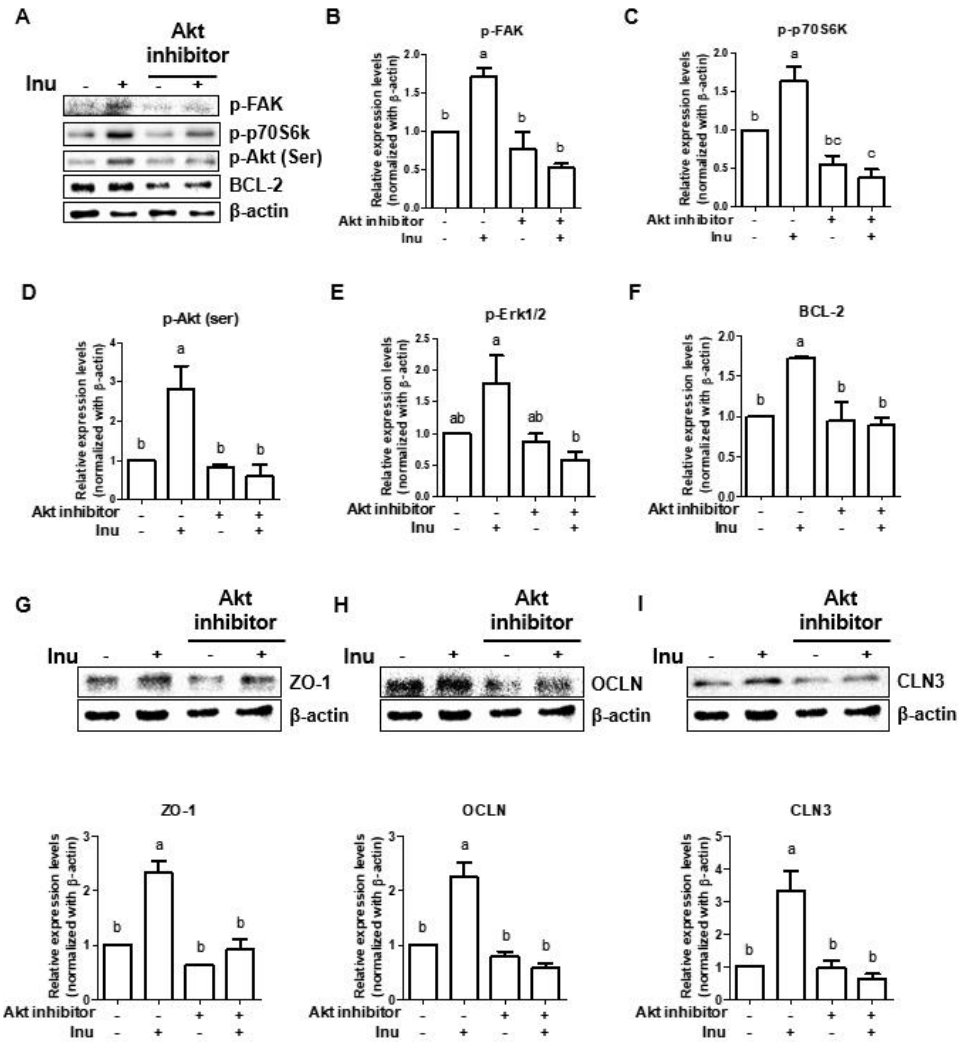

Figure 6. Inulin activates Akt signaling to increase the expression of tight junction proteins in IPEC-J2 cells. Confluent IPEC-J2 were stimulated with Akt inhibitor at 1 μM for 24 h prior to the treatment of inulin (Inu) at 1000 μg/mL for 24 h. (A, F-H) The whole cells were lysed, and protein extracts were analyzed by using Western blot assay. The relative

expression to the β-actin for (B) p-FAK, (C) p-p70S6K, (D) p-Akt (serine 473), (E) BCL-2, (F) ZO-1, (G) OCLN, and (H) CLN3 is expressed as mean ± SEM (n = 3). Different letters indicate significant differences at $P < 0.05$ between the groups.

4) Inulin prevents DON-induced barrier disruption in IPEC-J2 cells.

Next, the damage was induced in IPEC-J2 by DON treatment and then the protective effect of inulin was examined. Similar to the previous report [34], DON decreased the TEER (Fig. 7A), reduced cell viability (Fig. 7B), and diminished the expression ZO-1, OCLN, and CLN3 in a time- and dose-dependent manner (Fig. 7C).

Next, the prophylactic effect of inulin was examined. IPEC-J2 were pretreated with inulin for 24 h and treated with DON for 48 h, and then TEER, cell viability and the expression of tight junction proteins were examined. Inulin prevented the DON-induced decrease in TEER at 48 h (Fig. 7D). Furthermore, DON-induced cell death was significantly ($P < 0.05$) reduced in the cells pretreated with inulin (Fig. 7E). To further verify the effects of inulin under the DON-induced disruption, expression of the tight junction proteins was measured. Inulin-pretreated IPEC-J2 with damage induced by DON had higher protein levels of ZO-1, OCLN, and CLN3 than the cells treated with DON (Fig. 7F). This result suggests that inulin could directly alter the expression of tight junction proteins and protect against disrupted intestinal epithelium.

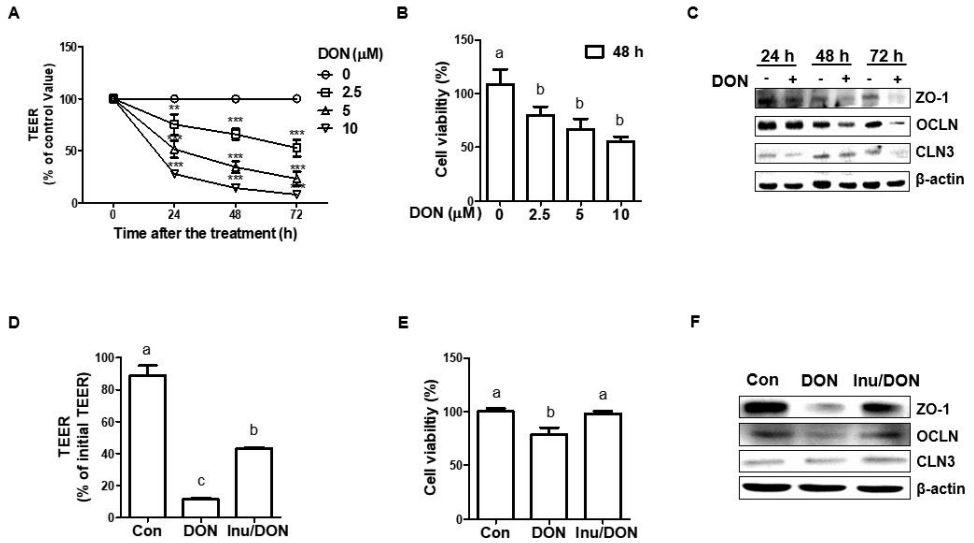

Figure 7. Inulin prevents DON-induced barrier disruption in IPEC-J2 cells. (A) Differentiated IPEC-J2 were treated with DON (0, 2.5, 5, or 10 μM). TEER values were measured using voltohm meter at 0, 24, 48 or 72 h. $^{**}P < 0.01$ and $^{***}P < 0.001$. (B) Confluent IPEC-J2 were treated with DON (0, 2.5, 5, 10 μM) for 48 h. Viability of the cells was examined by using MTT assay at 48 h after the DON treatment (n = 3). (C) Confluent IPEC-J2 were treated with 5 μM of DON for 24, 48 or 72 h. Whole-cell lysates were analyzed for the expression of ZO-1, OCLN, CLN3, and β-actin by using Western blot assay. The representative figure from three similar results is shown. (D) Differentiated IPEC-J2 were pretreated with 0, 10, 100 or 1000 μg/mL of inulin (Inu) for 24 h, and then treated with DON (5 μM) for 48 h. TEER value was measured at 48 h, represented as

percentage of initial TEER (n = 3). (E-F) Confluent IPEC-J2 were pretreated with 1000 μg/mL of Inu for 24h and treated with DON (5 μM) for 48 h. (E) Viability of the cells was examined by using MTT assay (n = 9). (F) Protein levels of ZO-1, OCLN, and CLN3 at 48 h after DON exposure were examined from the lysates by Western blot assay. β-actin was used as an internal control. All data shown in B, D and E are expressed as means ± SEM. Different letters indicate significant differences at $P < 0.05$ between the groups.

5) Inulin alleviates DON-induced expression of IL-8 in IPEC-J2 cells.

Next, to examine whether inulin suppresses the DON-mediated enhancement of IL-8, the expression of IL-8 in mRNA and protein levels was examined by using *q*RT-PCR and ELISA, respectively. Notably, the expression of *IL-8* mRNA was significantly decreased in the inulin-pretreated cells prior to the damage induced by DON (Fig. 8A). Similarly, inulin pretreatment restored the protein expression of IL-8 to the level of control (Fig. 8B). These results demonstrated that inulin protects the damage of the cells resulting reduction of the pro-inflammatory cytokine, IL-8.

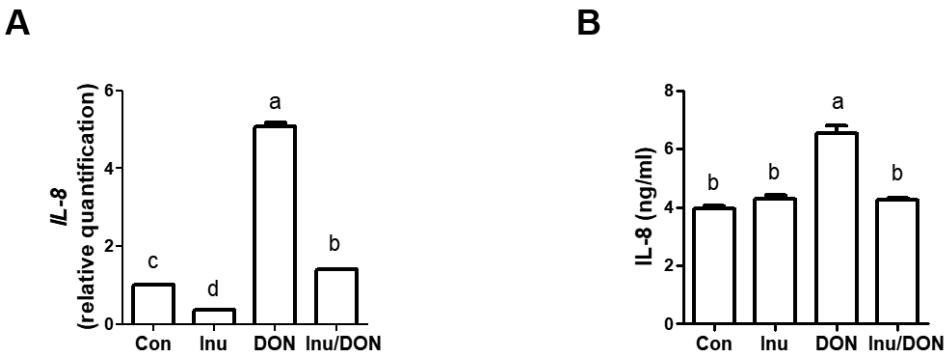

Figure 8. Inulin alleviates DON-induced expression of IL-8 in IPEC-J2 cells. Confluent IPEC-J2 were treated with 1000 μg/mL of inulin (Inu) (A) for 12 h (B) or 24 h, followed by DON (5 μM) treatment. (A) The cells were harvested and mRNA expression of *IL-8* was measured by real-time PCR (n = 3). (B) The supernatants were harvested and the level of IL-8 was measured by ELISA (n = 9). All data are expressed as means ± SEM. Different letters indicate significant differences at $P < 0.05$ between the groups.

V. Discussion

Feeding pigs with inulin as a feed additive has been shown to increase body weight gain and to improve intestinal health by increasing villous height in the jejunum [66, 69]. It has been reported that inulin critically strengthens the barrier integrity through increased composition of probiotics and their metabolites, SCFA in porcine IECs [2]. However, the functional and biological mechanism of inulin in the porcine IECs has not been studied in detail. Thus, the barrier disruption was induced by DON which leads to major economic problem in the porcine industry, because of low productivity and growth performance [75], and the effect of inulin was investigated. The present study demonstrated that (i) inulin strengthened the barrier function in IPEC-J2 cells; (ii) inulin-mediated tight junction protein was dependent on Akt signaling; and (iii) inulin had a prophylactic property of inulin under the DON treatment.

In accordance with these results, the previous study reported that inulin increased the TEER and protein level of OCLN in the human intestinal epithelial cells [72]. Therefore, inulin is probably involved in activation of the barrier function by enhancing the expression of tight junction proteins in

intestinal epithelial cells. However, inulin is still unclear on what signaling pathway is involved in the tight junction formation. Otherwise, Akt signal has been well characterized for the induction of tight junction proteins, regulation of survival and strengthens the intestinal barrier function [59, 60]. Previous study demonstrated that FAK in conjunction with Akt signals enhances the tight junction proteins and proliferation during the mucosal injury [56]. Furthermore, phosphorylation of Akt activates p70S6K which leads to increase of protein synthesis and proliferation in intestinal epithelial cells [57]. The downstream of Akt, BCL-2 has a role of preventing the apoptosis [58]. Therefore, depending on the previous studies, it was observed that inulin may have a relationship between Akt signal and tight junction proteins. In the present study, activation of Akt signaling induced the expression of tight junction proteins in epithelial cells when treated with inulin. It was confirmed through inhibition of Akt, which eradicated the enhanced barrier function on epithelial barrier integrity.

G-protein coupled receptors [76] and toll-like receptors (TLRs) [74] have been suggested as an upstream of Akt. The previous studies reported that inulin is recognized via TLR2, 4, 5, 7, and 8 to induce the activation of human embryonic kidney reporter cells [18, 77]. However, in a recent study, fructo-oligosaccharide (FOS), which has identical monosaccharide linkages

and differing only by length to inulin, activated TLR signaling. However, inulin could not in Caco-2Bbe1 cells [78]. It was noting in the present study that inulin does not link with TLR2 or TLR4 recognition (data not shown). Thus, it is not well-known which receptor exactly recognizes the inulin and that remains debatable.

DON significantly inhibited the expression of tight junction proteins along with increased membrane permeability of IPEC-J2 cells [34]. On the other hand, it has been identified to induce the activation of pro-inflammatory immune responses and to reduce the cell proliferation via MAPK/NF-κB and Akt signal pathway, respectively [37, 79]. Specifically, the other study showed that DON activates the MAPK signaling as well as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which induces apoptosis and pro-inflammatory gene expression [30], in IPEC-J2 cells [80]. The present results are supported by the previous studies [34]; DON treatment decreased the tight junction proteins and increased pro-inflammatory cytokines, IL-8. Moreover, the cell death was induced after DON treatment.

To overcome the detrimental effects of DON, inulin was pretreated to IPEC-J2 cells. Inulin mitigated the deleterious effects of DON by promoting the expression of tight junction proteins and inhibiting IL-8. Similarly, other

prebiotics study supported this present data, GOS diminished the harmful effects of DON on Caco-2 cells by strengthening the barrier function as well as prevents the expression of pro-inflammatory cytokines, IL-8 [81]. In terms of the inulin study, it showed inulin reduced of translocation of NF-κB in Caco-2 cells [16]. Additionally, in the case of *Escherichia coli* serotype O157:H7 (EHEC) challenge, inulin dampened the activation of MAPK and NF-κB in Caco-2Bbe1 cells. It was suggested that inulin may directly affect to signal transduction and hypo-responsiveness from inflammation [78]. Moreover, in this study, inulin pretreatment showed higher cell viability than DON treatment, suggesting the increased expression of BCL-2 may prevent the apoptosis. Therefore, it is likely that inulin may be an effective preventing agent for mitigating damage to IECs by DON via Akt signal in porcine intestinal epithelial cells. Nevertheless, further studies are needed to describe whether the protective effect of inulin against external mycotoxin in field trials can be applied.

In conclusion, this study demonstrated the action mechanism of inulin on porcine intestinal epithelial barrier function. It is possible that inulin may have a prophylactic effect against external mycotoxin. Therefore, based on the current findings, inulin may be used for feed supplementation to prevent the external infection in pig industry.

VI. Literature cited

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VII. Summary in Korean

유산균의 먹이(prebiotics)로 알려진 이눌린은 난소화성 다당류로 장내 균총을 변화시키고 이들로부터 생산된 대사물질인 단쇄지방산은 장내 항상성을 유도하는 것으로 알려진다. 대부분의 연구에서 돼지에게 이눌린을 사료첨가제 형태로 급이함으로써 그 효과를 규명하였지만, 이눌린이 장상피세포에서 미치는 세포 및 분자적 기전은 잘 알려지지 않고 있어 이에 대한 연구가 필요한 실정이다.

본 연구는 돼지 장상피세포에서 이눌린이 장벽강화기능을 가지며 곰팡이 독소 유래의 Deoxynivalenol (DON)에 의한 손상으로부터 장벽보호기능이 있음을 보여준다. 본 연구에서 사용한 이눌린의 24시간 처리는 세포 사멸과 세포의 분열에는 영향을 끼치지 않으면서 세포내 장벽강화를 유도했다.

이눌린은 tight junction (TJ) 단백질인 ZO-1, OCLN, CLN3의 단백질 발현을 증가시켜 장벽의 투과성을 줄였음이 확인되었으므로, 돼지 장상피세포 (porcine intestinal epithelial

cell-line, IPEC-J2) 에서 어떠한 기전에 의해 TJ 단백질의 발현이 증가하는지 확인하기 위해 관련 신호전달 물질을 관찰했다. 그 결과, 이눌린은 TJ 단백질을 증진시키는 신호전달 물질, Akt의 활성을 유도하였고, 이눌린을 통한 Akt의 활성을 억제하였을 경우, TJ 단백질 발현이 억제된다는 것을 관찰함에 따라 이는 Akt의 활성 유도에 의한 결과임을 확인할 수 있었다.

또한, 이눌린이 DON에 의한 장벽손상에 보호기능이 있는지 알아보기 위해 이눌린을 전 처리하였을 때 이는 DON으로 인해 증가되는 장관벽의 투과성을 억제하였고 세포사멸을 막으며, TJ의 붕괴를 억제할 뿐 아니라 염증성 사이토카인인 IL-8의 발현증가를 감소시킴을 확인하였다.

종합적으로, 본 연구는 DON에 의해 손상된 돼지 장상피세포에서 이눌린이 장벽보호가 Akt 기전을 통해 장벽보호기능을 향상시킨다는 것과 염증성 사이토카인을 줄일 수 있는 예방책으로서 기능이 있음을 보여주었으며, 효과적인 장벽보호제로서 이눌린의 이용 가능성을 제기한다.